A developmentally timed transgene excision system for somatic removal of gene editing components in asexually propagated plants

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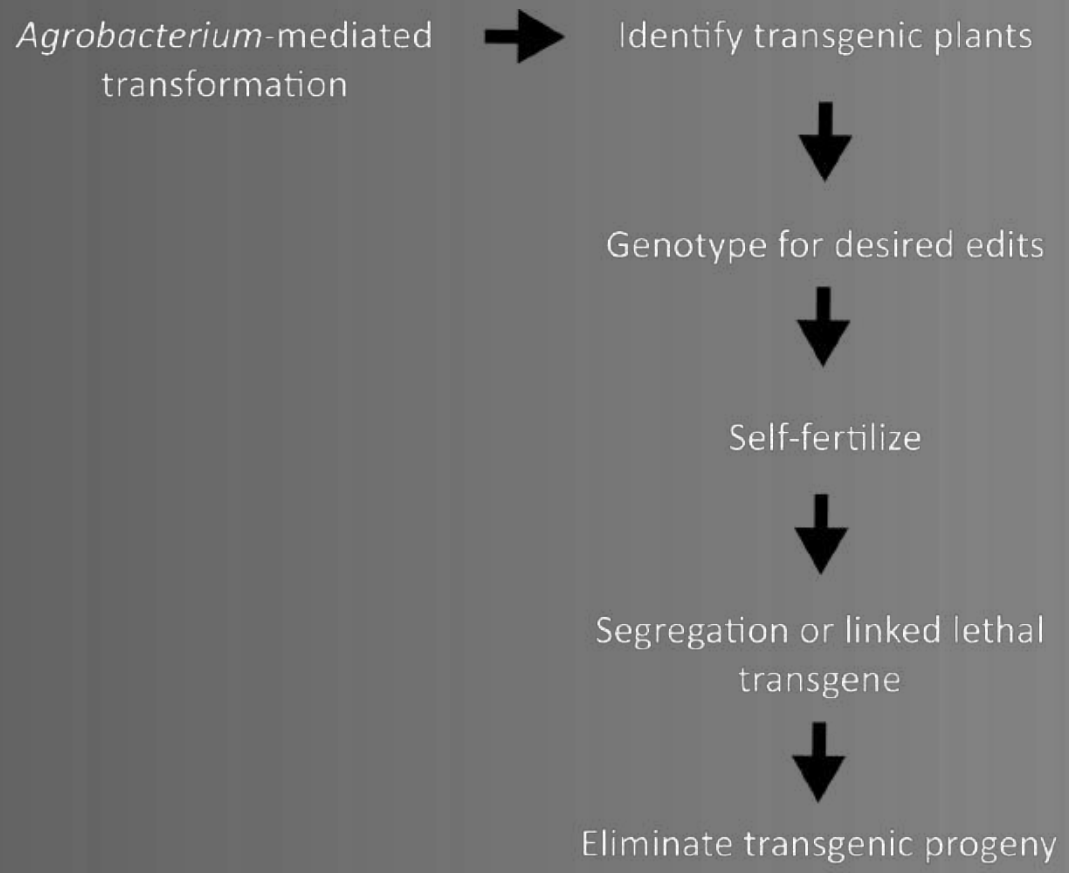
There are now abundant and simple CRISPR/Cas9 systems using stable transgene insertion



Eliminating transgenes with editing machinery is a problem in asexually propagated plants

* Wide interspecific crosses
* Intolerant to inbreeding
* Many years to maturity

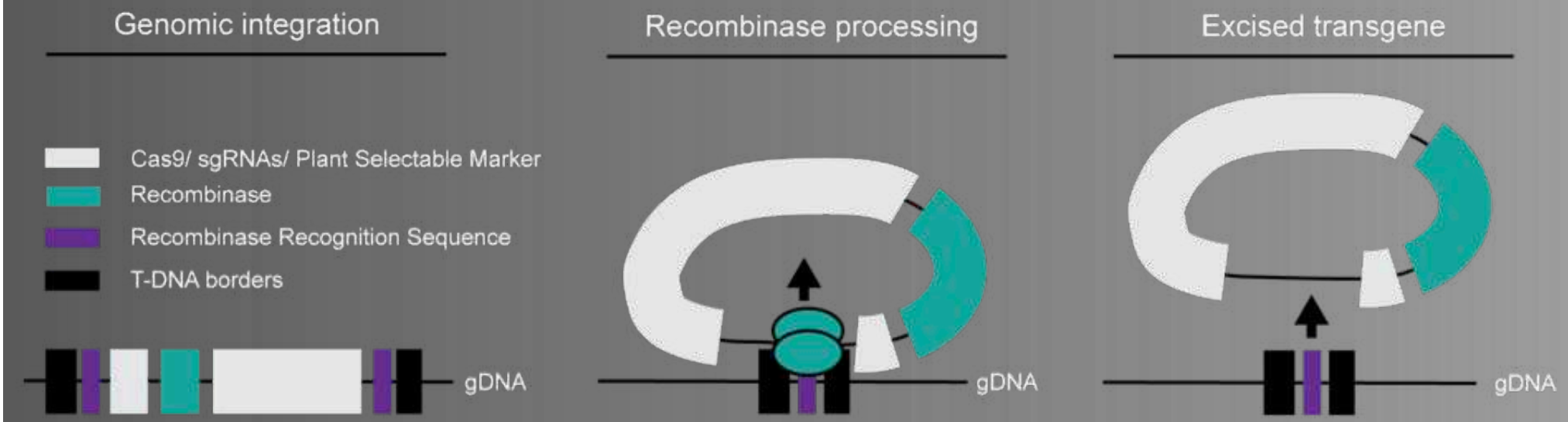
This is especially true for sterility traits for containment in short rotation forestry

- Transgene flow a major regulatory-market-public concern for GE trees (hybridization with wild relatives or exotic invasiveness)
- Gene editing of floral patterning genes may have much better containment than previous technologies (RNAi, pollen/seed ablation systems) – studies underway in *Populus* and *Eucalyptus* aimed at *LEAFY* and *AGAMOUS*
- Removal of gene editing components in these applications would require a somatic excision system unless transient delivery systems are used

Ongoing field trial of *leafy* and *agamous* mutant hybrid poplar near Corvallis, Oregon

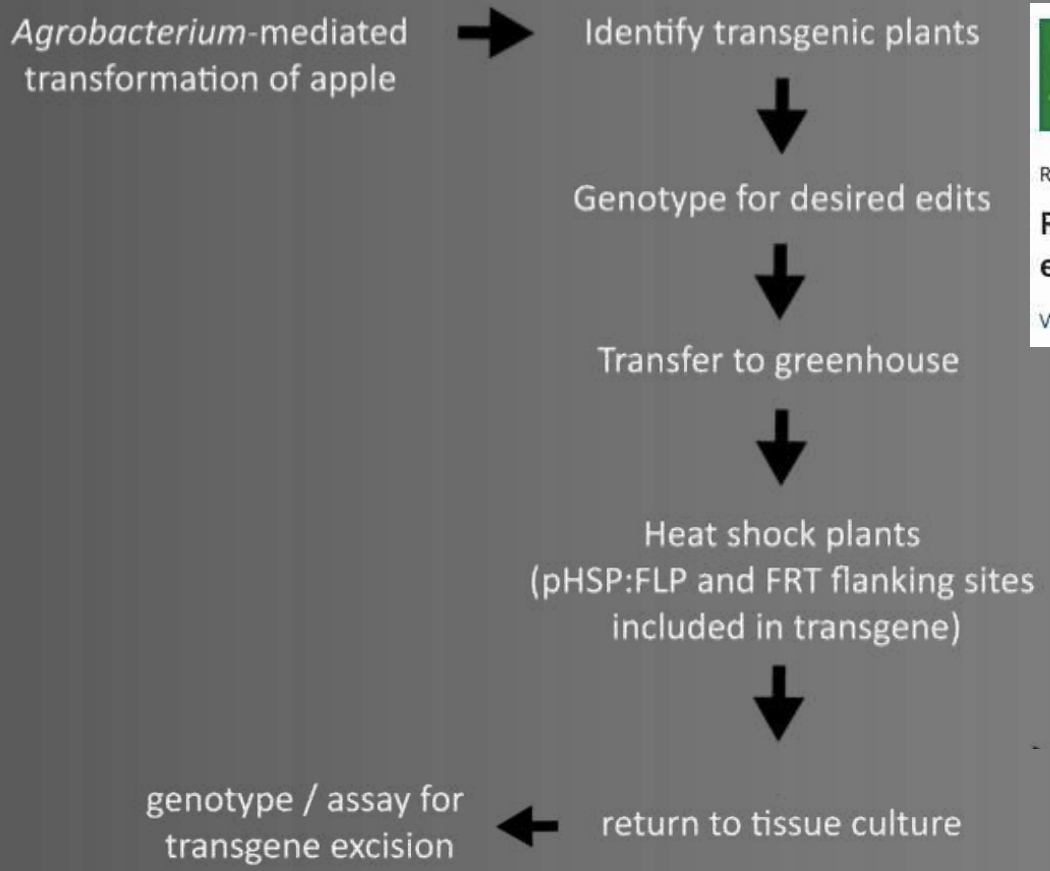
Estefania Elorriaga, PhD

Site-specific recombinases (SSRs) a long known tool for transgene removal - but never developed in to a reliable, efficient method for asexual excision



- Cre, FLP, R, others have been used for transgene excision in plants
- Efficient at excision over long distances
- High fidelity for recognition site

A related method recently published – but difficult to employ



Plant Biotechnology Journal

Research Article | Open Access

Reduced fire blight susceptibility in apple cultivars using a high-efficiency CRISPR/Cas9-FLP/FRT-based gene editing system

Valerio Pompili, Lorenza Dalla Costa, Stefano Piazza, Massimo Pindo, Mickael Malnoy

Pompili et. al., 2019 PMID: 31495052

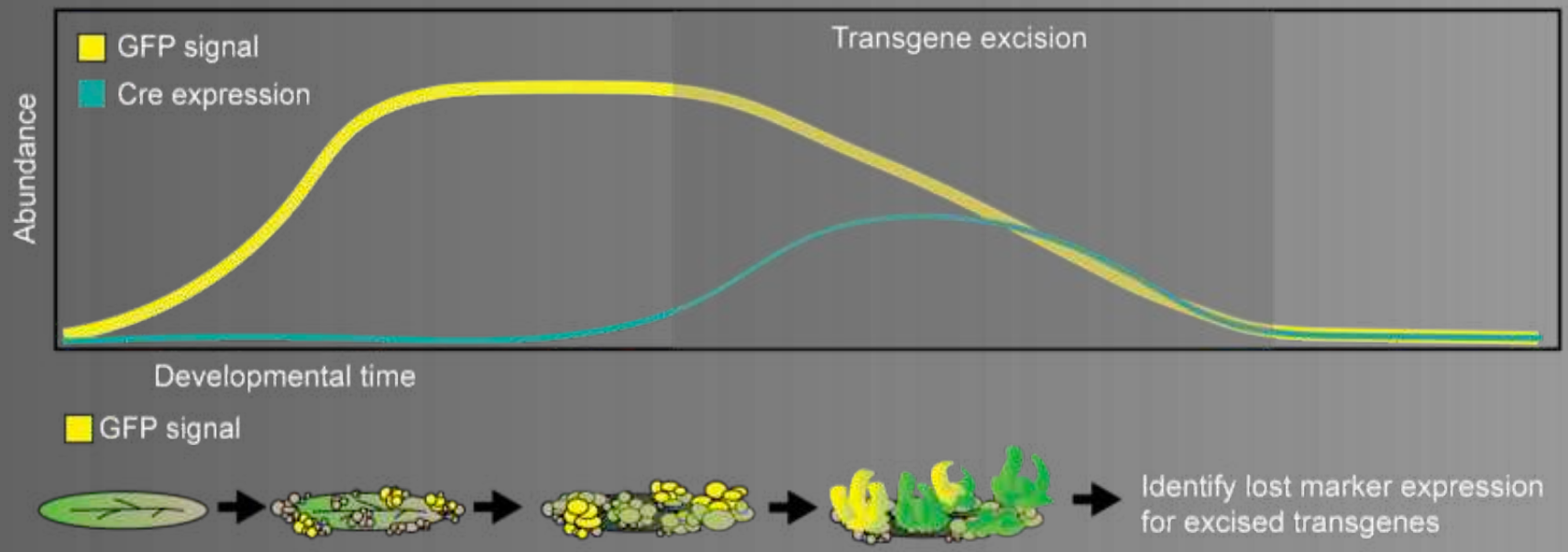
Concept for a developmentally regulated transgene excision system using SSRs

Evaluation of seven promoters to achieve germline directed Cre-*lox* recombination in *Arabidopsis thaliana*

Frédéric Van Ex, Dimitri Verweire, Martine Claeys, Ann Depicker & Geert Angenon

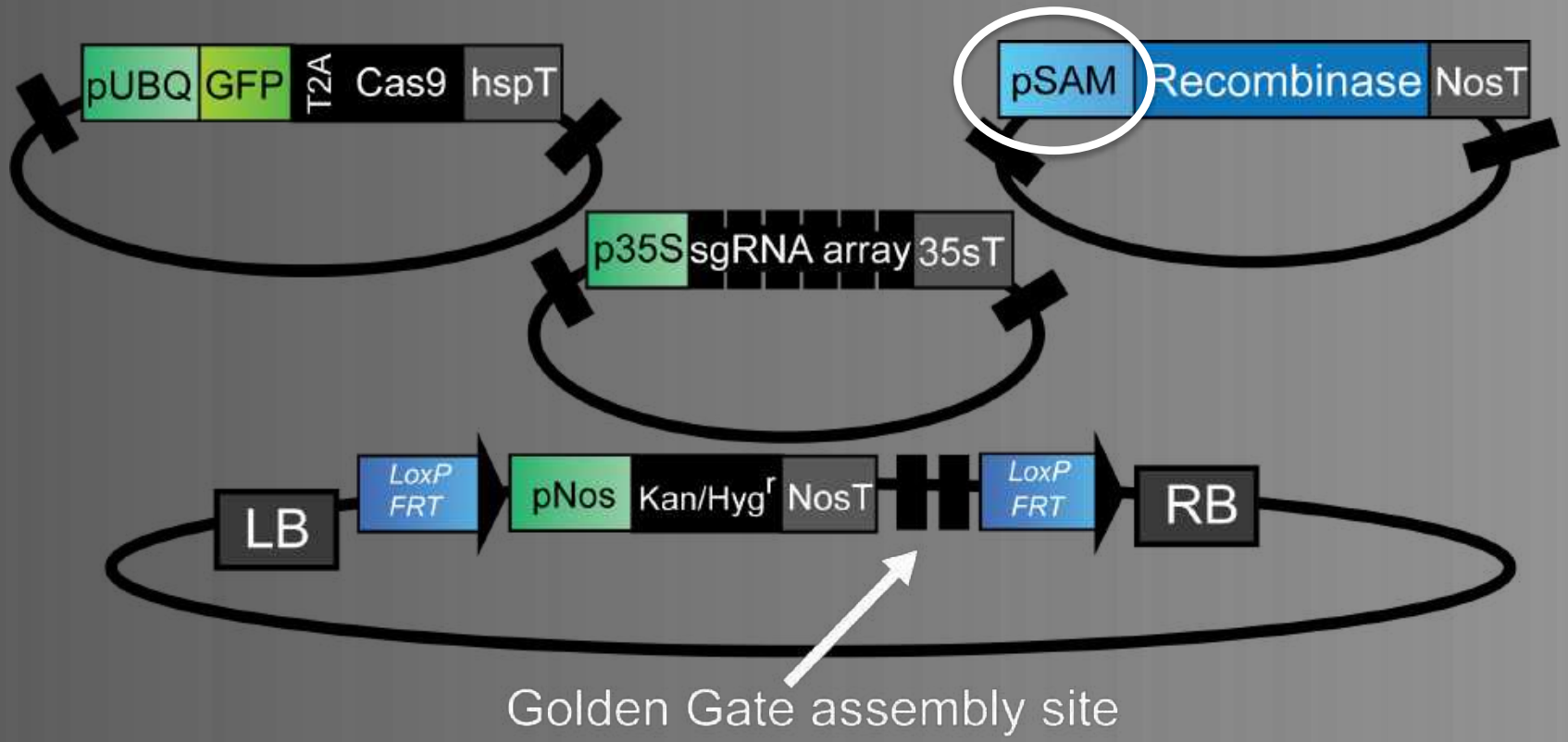
Plant Cell Reports **28**, 1509–1520(2009) | Cite this article

541 Accesses | **15** Citations | **0** Altmetric | Metrics



Excision is temporally and developmentally separate from the gene editing process

We cloned components into a widely used modular plant gene editing vector toolkit for facile assembly



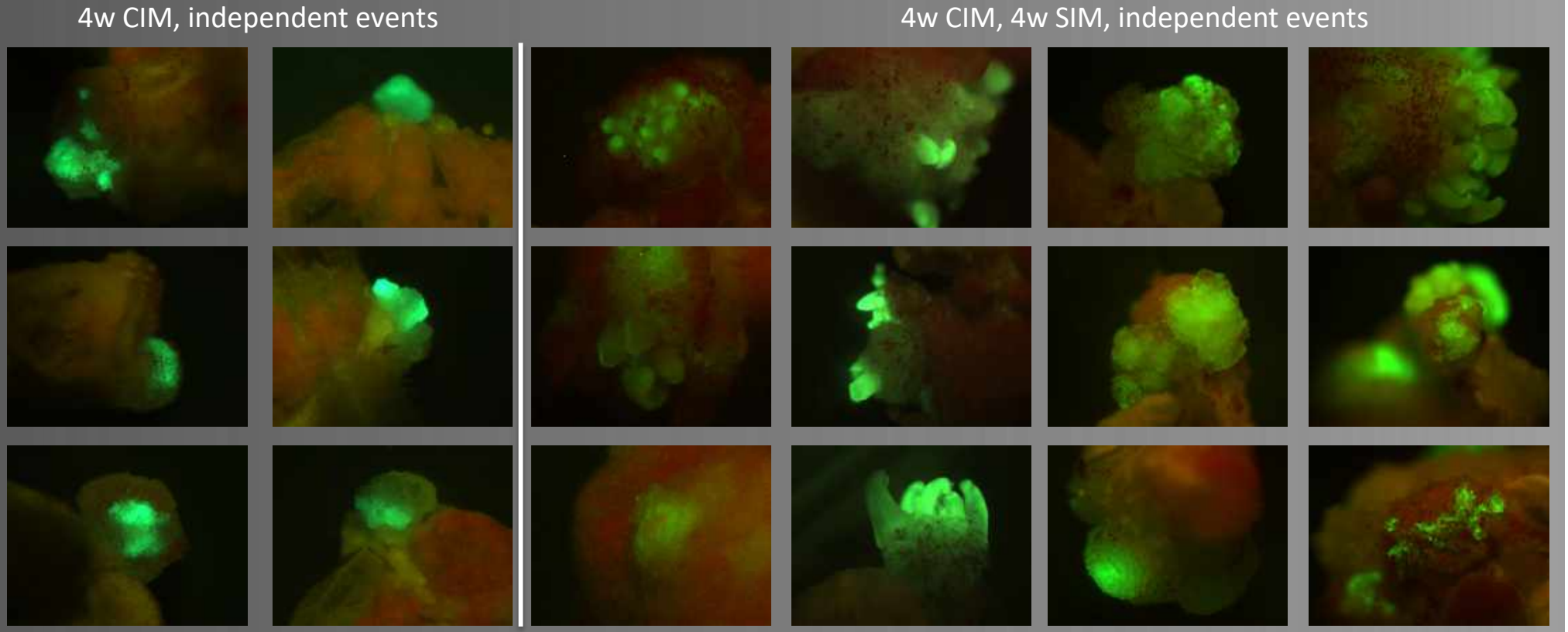
Čermak et. al., 2017 PMID: 28522548

Potential shoot meristem promoters to drive Cre were analyzed for tissue specific expression

Promoter	Length (from TSS)
AtWUS	2.2kb
PtWUS2	2.5kb
AtSTM	3.6kb
PtSTM	2.5kb/1.3kb
AtCSP3	1.3kb
PtCSP3	2.5kb
AtER	1.3kb
PtER	2.5kb
AtYAO	1.4kb
AtESR1/DRN	1.6kb
GmHSP17.5	450bp
AtUBQ10	1.3kb
PtUBQ10	1.5kb

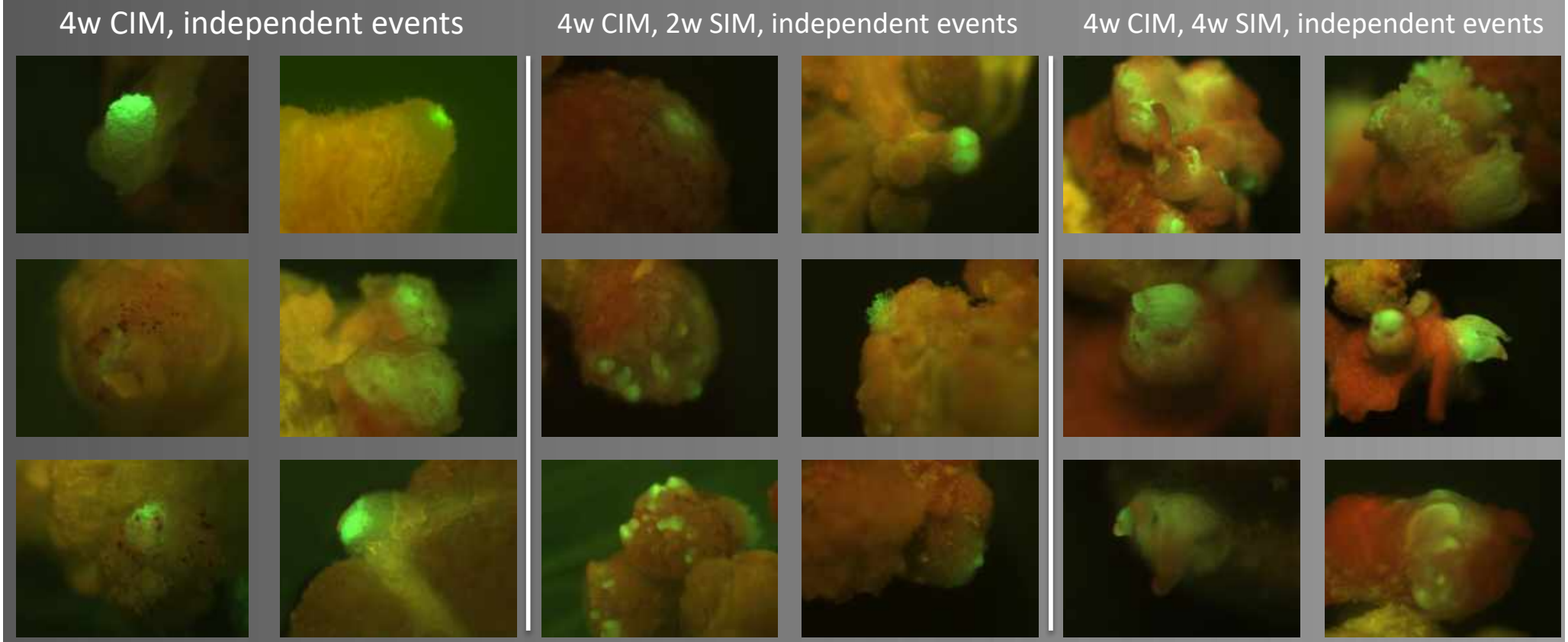
- Selected promoters from the literature known to be expressed in meristems or regeneration
- Avoided any genes with complex 3' dependent transcriptional regulation
- Cloned minimal Arabidopsis promoter fragments and *P. trichocarpa* putative homologs where applicable
- Used promoter:GFP constructs to perform non-destructive imaging during regeneration
- Average of 15 lines per construct produced, propagated for imaging and quantitative analysis of mean and variance in cell specific expression

Arabidopsis *COLD SHOCK PROTEIN 3 (CSP3)* promoter shows strong expression in regenerating callus and shoot meristems of poplar



- 1.3kb fragment upstream of the *AtCSP3* TSS is first expressed in late callus development and is highly expressed in shoot primordia
- Note some variation in expression among insertion events

The Arabidopsis *SHOOT MERISTEMLESS (STM)* promoter is weaker, shows higher variation in regenerating callus and shoot meristems



- 3.6kb fragment upstream of the *AtSTM* TSS is first expressed in late callus development and is highly expressed in shoot primordia
- Note lower and more restricted expression compared to *AtCSP3*; also high variance

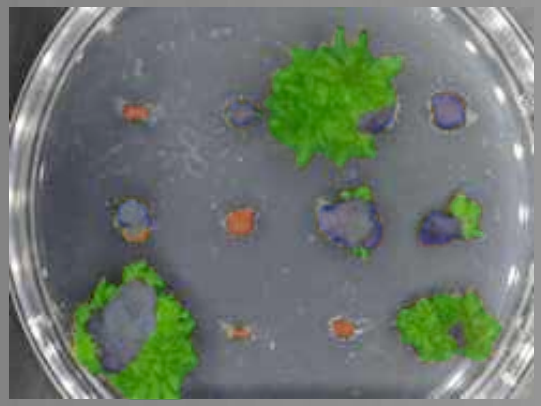
Quantification of event to event expression changes will aid in promoter choice

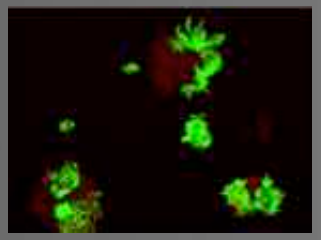
Michael Nagle, PhD Candidate

RGB image

Deep segmentation

Segmented image

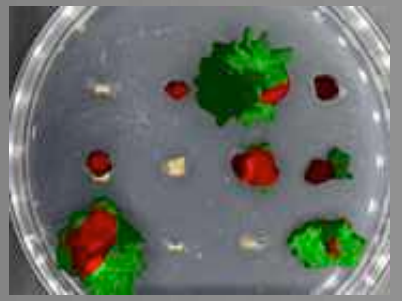




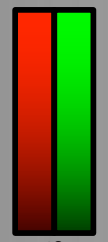
Hyperspectral image

Regression

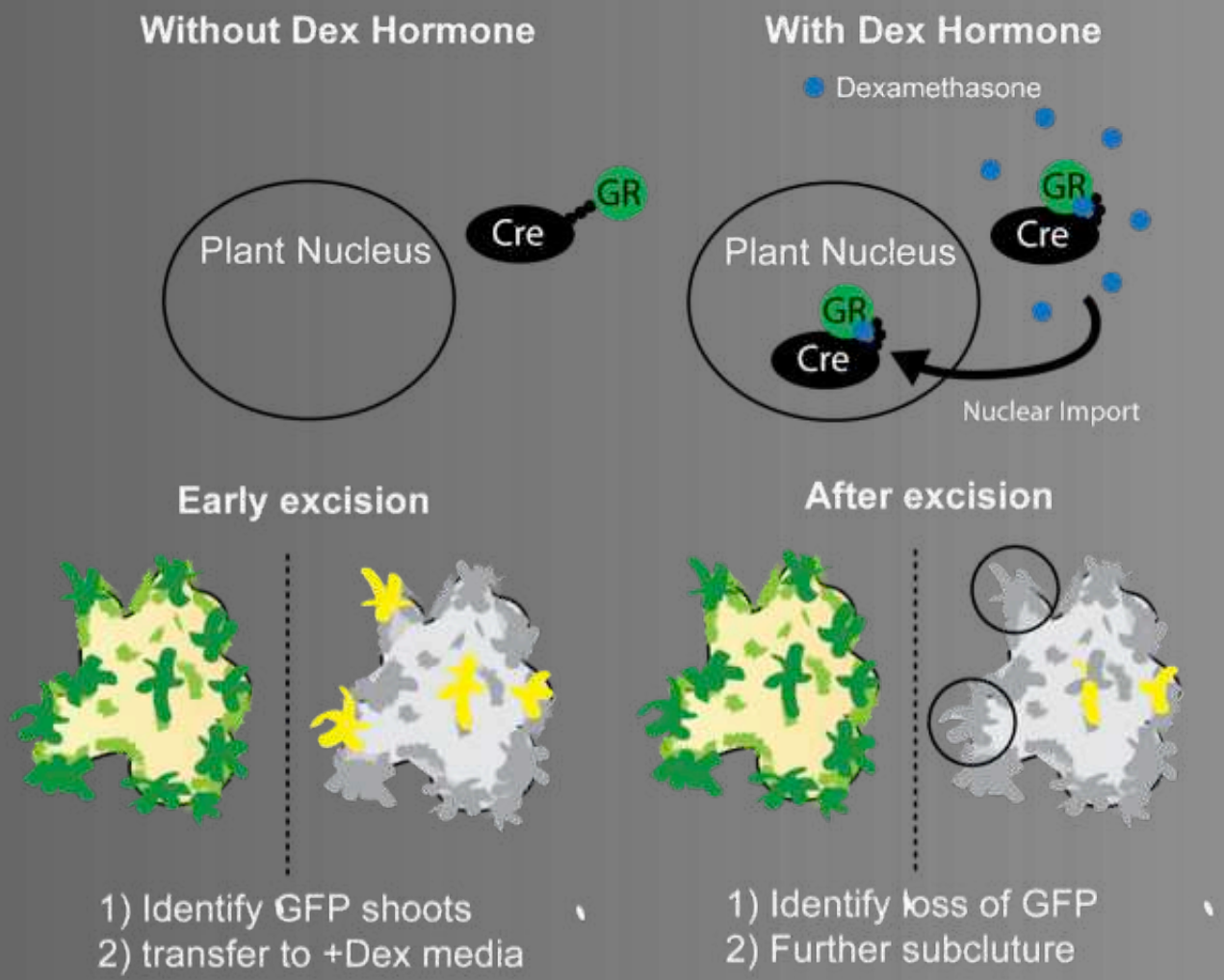
Protein fluorescence in specific tissues



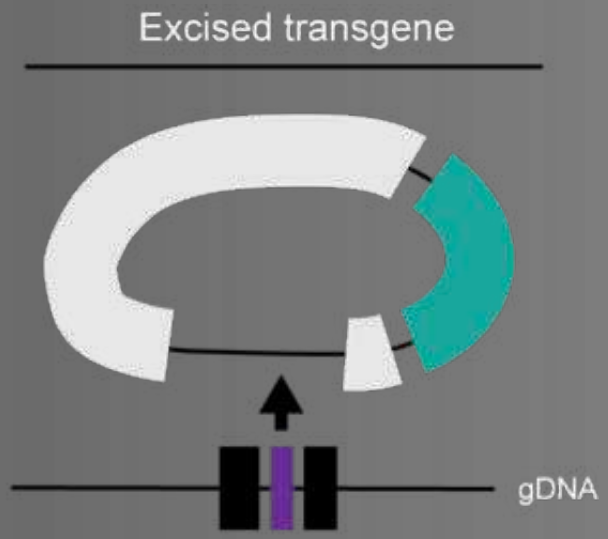
T-statistics for GFP



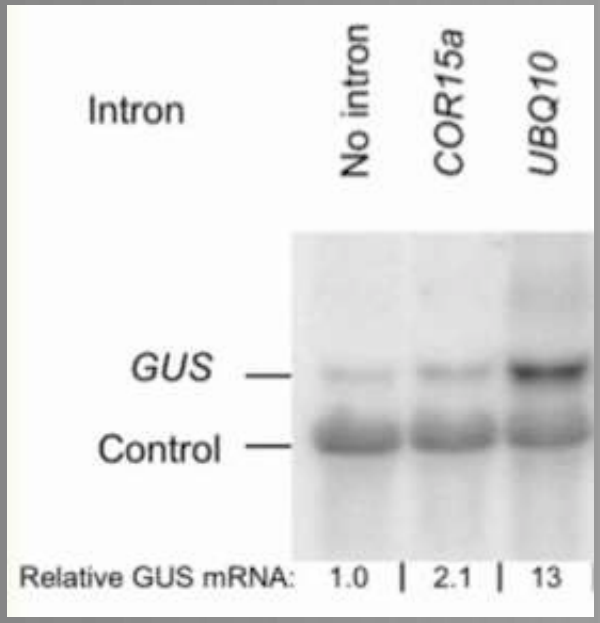
A fusion of Cre recombinase to a glucocorticoid receptor (GR) was chosen for two levels of control



Intron choice essential to prevent bacterial excision and avoid intron-mediated enhancement (IME)



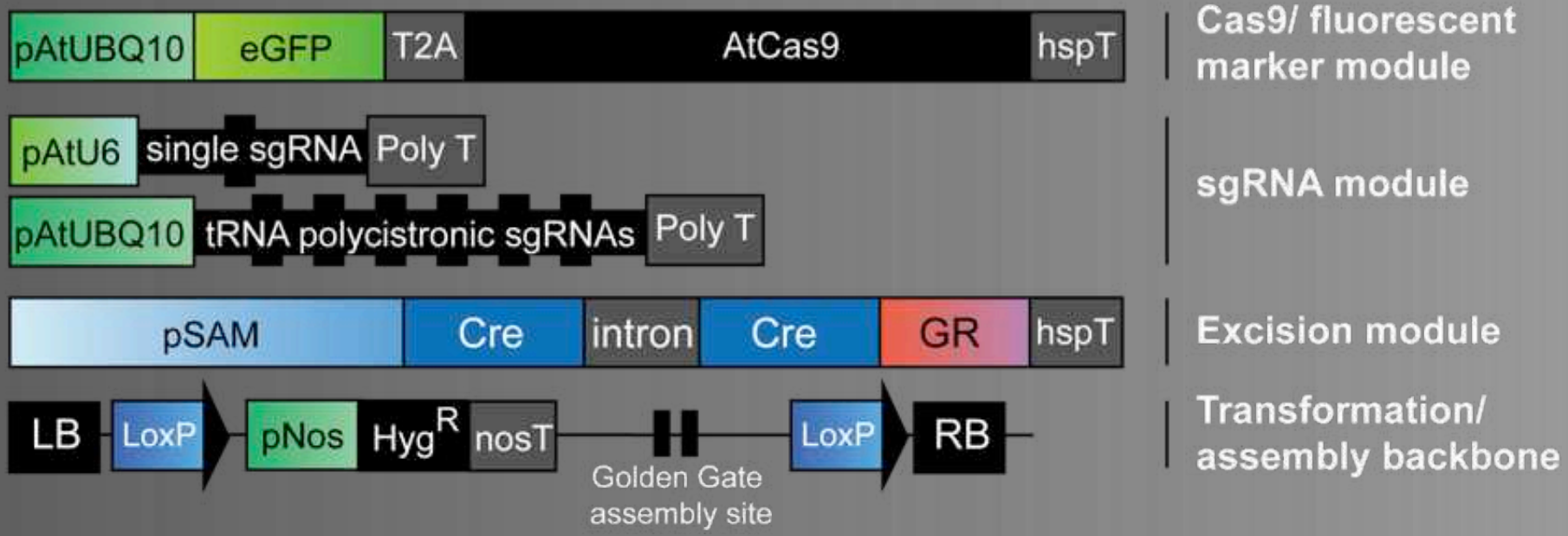
235bp excision “footprint”



Gallegos and Rose, 2017 PMID: 28373518

- Observed complete excision in *E. coli* when Cre construct was included without intron in 100% of colonies…….
- We included the Arabidopsis *COR15a* intron to reduce the potential for IME
- We placed the intron at the same codon position as a previously reported Cre gene
- Codon optimized for dicot expression (gene synthesis)

Current vector designs for editing and excision



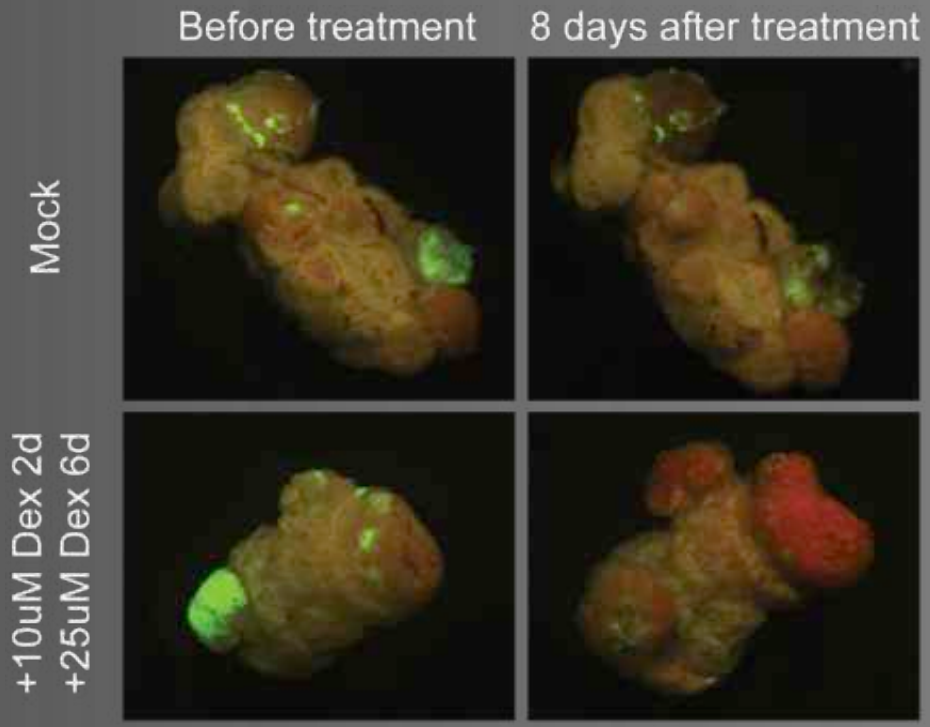
eGFP/Cas9 bicistronic transcript functional for identification of transgene insertion and editing

- *pAtUBQ10:GFP-T2A-Cas9* gene has easily visualized fluorescence under stereoscopic low magnification
- We edited the *PHYTOENE DESATURASE (PDS)* gene to visually check the editing rate after transformation, and confirmed Cas9 function when expressed as a bicistronic transcript with GFP

Initial testing of gene editing and developmental excision system in hybrid poplars (717-1B4)

- Target for editing:
  - *PHYTOENE DESATURASE (PDS)* – chlorosis as editing marker
- One Cre construct was used: *pAtCSP3:dsRed2-T2A-Cre-GR*
- Plants kept on selective media for 4 weeks on CIM, then transferred to SIM for 3 weeks, then transferred to SIM + 50uM DEX or mock treatment without selection
- *pds* transformation subset closely observed during early stages of DEX induction beginning after 3 weeks of CIM

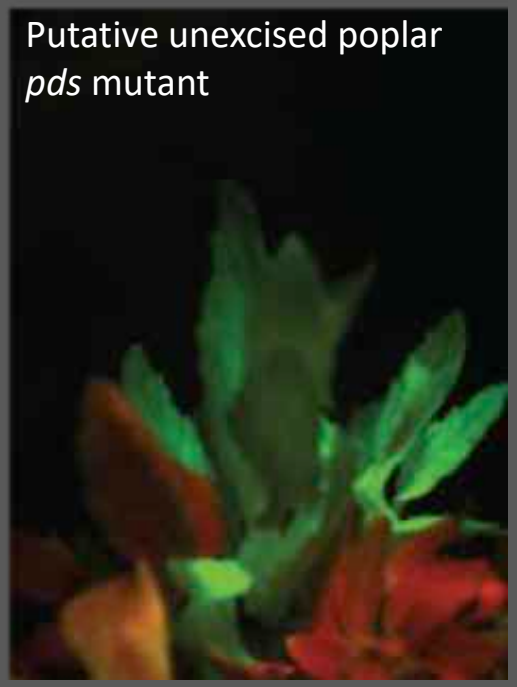
Fluorescent observation of early DEX treatment shows the potential for transgene excision

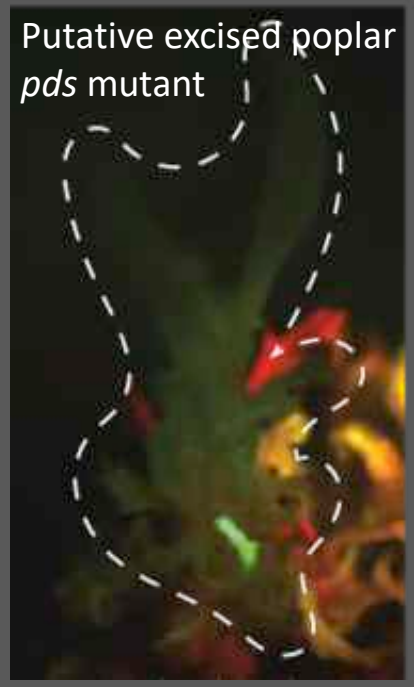


	Decreased GFP	No GFP	No change
Mock (n=15)	20%	0%	80%
+Dex (n=15)	53%	13%	33%

- DEX treatment was supplied in the media
- We observed a decrease of GFP expression in transgenic callus tissue after DEX treatment
- We were unable to observe dsRed expression from the *Cre* gene (T2A peptide/bicistronic transcript)

Limited loss of fluorescence observed with late shoot regeneration after DEX application in *pds* mutants





- Isolated 51 chlorotic *pds* mutants from DEX treated population
- 26% (n=40/150) of explants contained an edited, chlorotic shoot
- 4 events (8% of chlorotic shoots) showed an apparent elimination of GFP signal in regenerated shoots
- Studied both GFP and non-GFP excision to understand dynamics

Ongoing experiments to determine dynamics of gene editing and excision with DEX treated *pds* mutants

- Currently genotyping both edited and non-edited shoots for the excision footprint and components of the transgene
- Qualitative experiments suggest incomplete excision in non-fluorescent edited plants, and non-edited plants have detectable transgenic tissues
- This could mean Cre activity is too low, or overactive excision before gene editing is complete in many lines – we aim to test these in subsequent experiments
- Quantitative assessments of transgene loss currently underway to understand of editing and excision

Future directions

- Poor excision rate in edited lines suggest changes are still required for a reliable system in asexually propagated crops
- Quantification of promoter:GFP events, insulator testing, and screening new promoters may yield improved Cre expression cassettes
- Hyperspectral/deep segmentation system will aid high throughput analysis of DEX effects and variation thereof
- Once a robust system is in place, we aim to test in other asexually propagated crops (e.g., mint/hops)

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